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METABOLITE-INDUCED ACTIVATION OF HEPATIC PHOSPHOFRUCTOKINASE

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SUMMARY: Hepatic phosphofructokinase, isolated in a medium containing 100 mM $(NH_4)_2SO_4$, can be activated by ATP. This metabolite-induced activation was investigated in view of the suggestion that it is related to phosphorylation of phosphofructokinase. The results obtained do not support this interpretation. Inhibitors of protein phosphatases (NaF) and kinases (the Mg^{++}-chelator, ethylene diamine tetraacetic acid) did not affect the recovery of phosphofructokinase. In contrast, media of high ionic strength reduced the phosphofructokinase activity and rendered the enzyme sensitive to ATP-induced activation. Activation was also induced by other known effectors of phosphofructokinase (nucleoside triphosphates, fructose bisphosphates) and was not dependent on Mg^{++}-ions. It is suggested that activation represents ligand-induced reversal of the inactivation of phosphofructokinase which occurs at high ionic strength. The differential sensitivity of phosphofructokinase from fed or starved animals to inactivation and reactivation is discussed.

INTRODUCTION: Hepatic phosphofructokinase (6-phosphofructo 1-kinase, EC 2.7.1.11; PFK) occupies a key position for the control of liver carbohydrate metabolism and its activity is modulated by several metabolites (including substrates, products, and the recently discovered activator, fructose 2,6-bisphosphate) (1-3). PFK is also subject to phosphorylation by cyclic AMP-dependent protein kinase, although the significance of this modification of the enzyme for its physiological regulation remains unclear (4-6).

Brand and Söling (7-9) have described an ATP- and time-dependent activation of PFK in rat liver supernatants prepared in the presence of phosphate and ammonium sulphate. The degree of activation varied with the nutritional state of the animal. The activation was ascribed to phosphorylation of PFK by an endogenous cyclic AMP-independent protein kinase. The case for this putative phosphorylation mechanism has however remained unproven, and this report describes experiments designed to clarify this question. The results do not support the contention that ATP-induced activation is mediated by protein phosphorylation and show that other metabolites can

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induce a similar activation of PFK. It is clear however that the susceptibility to metabolite-induced activation of PFK differs in supernatants derived from livers of fed or starved animals. The likely cause of these differences is discussed.

MATERIALS AND METHODS: Coupling enzymes and NADH were from Boehringer (Lewes, England) and other biochemicals were from Sigma (Poole, England) and of the highest grade available. Rats (CD-strain) were kept on a 12h dark/light cycle (1700, 1900) and allowed free access to food and water. Where appropriate, food deprivation was begun at 0900. All animals were sacrificed by cervical dislocation between 0830 and 0900.

Livers were rapidly removed, chilled on ice and homogenised in a Teflon/glass homogeniser in Medium A (20 mM potassium phosphate, 100 mM $(NH_4)_2SO_4$, 0.5 mM $MgCl_2$, 5 mM β-mercaptoethanol, pH 7.6 (7)) unless otherwise indicated. The homogenate was centrifuged at 75 000 x *g* for 40 min at 4° and an aliquot was gelfiltered on a Sephadex G-25 column in 20 mM potassium phosphate, 0.5 mM $MgCl_2$, 5 mM β-metcaptoethanol, pH 7.6. The main protein-containing fraction was used in the subsequent experiments.

Phosphofructokinase activity was determined under optimal conditions as described by (10). Where appropriate, preincubation of samples prior to assay was at 20°C for 10 min unless otherwise stated. Protein was determined as in (11).

RESULTS: When rat livers were homogenised and fractionated in Medium A (8) a substantially higher PFK activity was detected in gelfiltered supernatants from fed as compared to 24h-starved animals. Preincubation of the gelfiltrate with 5 mM ATP resulted in a substantial increase in the PFK-activity measured under optimal conditions, the final activity now being similar for fed or fasted animals, in agreement with (8). (Table I). Activation by ATP was maximal after 5 min preincubation and the half-maximally effective ATP concentration was ∿2 mM (Fig.1). Further experiments were performed using homogenisation media in which phosphate was replaced by Tris-HCl, ammonium sulphate by NaCl or NaCl plus NaF (protein phosphatase inhibitor) or in which $MgCl_2$ was replaced by the chelating-agent EDTA. The results showed (a) a substantially higher basal PFK activity than in samples prepared using Medium A and (b) little or no effect of preincubation with ATP (Table I). Only when medium containing $(NH_4)_2SO_4$ was used was substantial ATP-activation observed. To test whether this was due to $(NH_4)_2SO_4$ itself or to its relatively high ionic strength, media containing high KCl concentrations the measured PFK actively fell, but the enzyme could be activated to a greater extent by ATP-preincubation (Table 1). An identical time-course

TABLE 1
PFK Activity in Gelfiltered Supernatants from Fed or Starved Rats

Medium	PFK Activity mU mg protein^{-1}					
	24h-fasted			Fed		
	-ATP	+ATP	Ratio $\frac{+ATP}{-ATP}$	-ATP	+ATP	Ratio $\frac{+ATP}{-ATP}$
A: (8)	5.03+0.5 (7)	24.6+2.2 (7)	4.9	11.3+1.0 (7)	23.8+1.5 (7)	2.1
B: 20mM Tris-HCl, 0.1M KCl, 0.5mM $MgCl_2$, 5mM β-mercaptoethanol, pH7.6	10.7+0.4 (3)	11.9+1.8 (3)	1.1	19.7+2.9 (3)	19.0+4.4 (3)	0.96
C: 20mM Tris-HCl, 0.05M KCl, 0.05 M KF, 0.5mM $MgCl_2$, 5mM β-mercaptoethanol, pH7.6	9.6+0.7 (3)	10.6+0.7 (3)	1.1	16.7+1.2 (3)	18.5+0.8 (3)	1.1
D: 20mM Tris-HCl, 0.5M $MgCl_2$, 0.1 M $(NH_4)_2SO_4$, 5mM β-mercaptoethanol, pH 7.6	6.4+0.2 (3)	24.6+2.1 (3)	3.9	N.D	N.D	N.D
E: as B, but 0.2 M KCl	10.1+1.3 (5)	14.2+1.8 (5)	1.4	12.7+2.3 (3)	14.9+3.2 (3)	1.2
F: as B, but 0.4 M KCl	2.6+0.3 (3)	22.2+4.9 (3)	8.6	5.0+2.7 (3)	23.5+3.3 (3)	4.7

Livers were homogenised in the medium indicated, and PFK-activity was determined after preincubation of gelfiltered supernatants without or with ATP (5 mM). Results are expressed + standard deviation and the number of experiments in each case is indicated in parentheses.

and concentration-dependence was shown for ATP-mediated activation of PFK in samples prepared in Medium A or in high KCl (not illustrated).

Table 2 illustrates that activation by ATP persisted after further gelfiltration on Sephadex G-25 and did not require Mg^{2+}-ions (although they

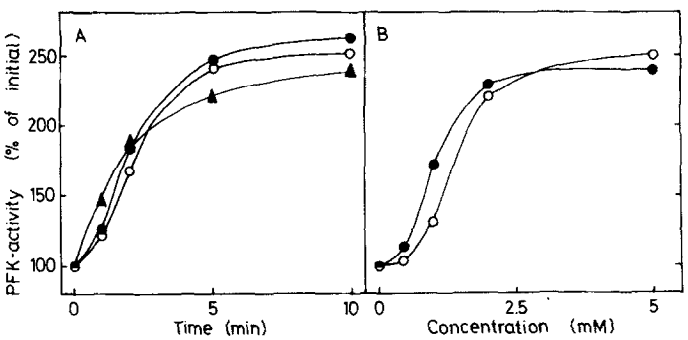

Fig.1. Activation of PFK by ATP (O), fructose 1,6-bisphosphate (O) or fructose·2,6-bisphosphate (Δ). Gelfiltered supernatants of liver (from 24h-starved rat, homogenised in medium A) were preincubated at 20° with (A) 5 mM ATP, 2 mM fructose 1,6-bisphosphate or 2 mM fructose 2,6-bisphosphate for the times shown or (B) with a range of ATP or fructose 1,6-bisphosphate concentrations for 10 min, prior to assay of PFK under optimal conditions. Fructose 2,6-bisphosphate was effective at μmolar concentrations (see Results).

TABLE II
Activiation of PFK by Adenine Nucleotides

Preincubation Condition	PFK-Activity mU mg protein^{-1}			
	Expt.1 (Fed)	2 (Starved)	3 (Starved)	4 (Starved)
H_2O	11.0	6.1	5.0	8.4
ATP (5 mM)	24.2	23.4	21.1	24.2
ATP (5 mM), then gelfiltered	21.3	20.5	18.4	21.8
ADP (5 mM)	11.9	7.9	6.0	10.5
AMP (5 mM)	9.7	6.2	5.1	9.8
ATP (5 mM) + EDTA (4 mM)	20.8	21.5	19.0	19.6
ATP (5 mM) + $MgCl_2$ (5 mM)	25.2	23.9	22.8	24.9

Livers were homogenised in Medium A.

enhanced the activation). Slight but variable activation was obtained with ADP, but AMP was ineffective. Other nucleotide triphosphates tested also activated PFK in preincubations, GTP and CTP being approximately as effective as ATP, UTP rather less so (Table III). Fructose 6-phosphate activated only weakly, but fructose 1,6-bisphosphate or fructose 2,6-bisphosphate was as effective as ATP. Activation occurred over a similar time course to that for ATP (Fig.1). Fructose 2,6-bisphosphate was effective at micromolar concentrations (half maximally effective concentration, ~8 μM) whilst millimolar concentrations of fructose 1,6-bisphosphate were required (Fig.1).

TABLE III
Metabolite-induced Activation of PFK

Metabolite	Concentration mM^{-1}	Relative Efficiency of Activation (Activation by Metabolite/Activation by ATP) x 100 (± Standard deviation)	
ATP	5	100	
GTP	5	85 ± 9	(17)
CTP	5	100 ± 16	(5)
UTP	5	65 ± 16	(5)
Fructose 6-phosphate	2	4,13	
Fructose 1,6-bisphosphate	2	100 ± 3	(9)
Fructose 2,6-bisphosphate	2	91 ± 12	(11)
ATP plus Fructose 1,6-bisphosphate	5) 2)	111 ± 6	(3)
ATP plus Fructose 2,6-bisphosphate	5) 2)	109 ± 7	(4)

Livers were homogenised in Medium A. The number of experiments in each case is shown in parentheses.

Activation by ATP and the fructose bisphosphates was not additive and activation of PFK by GTP or fructose 2,6-bisphosphate persisted after gelfiltration of the activated sample (not illustrated). Similar activation by ATP, nucleoside triphosphates and the fructose bisphosphates was observed in ten experiments in which livers were homogenised in Medium A or a medium containing 0.4 M KCl (Medium F of Table I).

DISCUSSION: The present results concerning the ATP-induced activation of PFK confirm and extend those of Brand and Söling (7,8) who ascribed the large differences in PFK-activity of supernatants from livers of animals of differing nutritional status, and the susceptibility of PFK to activation by ATP, to the ability of Medium A to maintain PFK in a phosphorylation state resembling that *in vivo*. It was suggested that $(NH_4)_2SO_4$ inhibited the (putative) PFK-kinase and phosphate inhibited the corresponding phosphatase. Our results do not support this interpretation since use of media containing NaF (phosphatase inhibitor) and EDTA (chelating agent for Mg^{2+}, required cosubstrate for protein kinases) did not reproduce the results obtained with Medium A. Instead, the effect of Medium A appears to be related to an inactivation of PFK occurring at high ionic strengths since media containing high KCl concentrations generate a similar loss of PFK-activity and enhanced susceptibility to activation by ATP. Several other lines of evidence suggest activation of PFK by ATP does not reflect a protein phosphorylation process; (i) PFK is activated equally effectively by several nucleoside triphosphates, whilst protein kinases are generally specific for ATP as phosphate donor and also exhibit a much lower Km than that apparent here (reviewed in 12,13), (ii) nucleoside triphosphate-induced activation did not require Mg^{2+}-ions, whilst NTP-Mg is the normal substrate in phosphotransfer reactions (12) and (iii) similar activation, which was not additive to that induced by nucleoside triphosphates, was mediated by the fructose bisphosphates, established effectors of PFK. It therefore appears likely that the metabolite-induced activation of PFK is due to a ligand-induced structural change in the enzyme rather than to its phosphorylation. It is

well-known that PFK undergoes complex oligomerisations, which are influenced by metabolites (5,14) and Brand *et al.* themselves showed ATP-activation is accompanied by association of PFK (8). The incorporation of ^{32}P-labelled phosphate from ATP into immunoprecipitable PFK (9) may reflect the presence of the catalytic subunit of cyclic AMP-dependent protein kinase in the PFK preparation used (15).

The difference in susceptibility of PFK from fed and starved animals to high-salt induced inactivation and thus to metabolite mediated activation may reflect either a modification to the enzyme itself or changes in cellular metabolite concentrations. In preliminary experiments, near-physiological concentrations (μmolar, (16)) of fructose 2,6-bisphosphate were found to protect PFK against inactivation. The intracellular concentration of this metabolite in liver falls precipitously in fasting (fed : 8 nmol g^{-1}; 18h-starved : 0.3 nmol g^{-1} (15)). The elucidation of the structural basis of metabolite-induced activation of PFK and its possible role in the physiological regulation of the enzyme requires further work, both with the purified enzyme and *in vivo*.

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